IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.	:	07/402,450	Confirmation No.: 8131
Applicant	:	George J. MURAKAWA et al.	
Filed	:	1 September 1989	
TC/A.U.	:	1631	
Examiner	:	Marina Miller	
Attorney Docket No.	:	2124-154	
Customer No.	:	6449	

Director of the United States Patent
and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.131(a)

Dear Sir:

We, George J. MURAKAWA, R. Bruce WALLACE, John A. ZAIA and John J. ROSSI, applicants for the above-identified patent application, declare as follows:

1. That some time on or prior to 21 August 1989, we conceived the idea of quantifying target viral RNA using a known amount of a reference RNA sequence as an internal control. That is, a reference RNA sequence, such as a maxigene or a minigene (each of which contain target viral RNA sequence), was added to the sample containing the target viral RNA. The target viral RNA and the reference RNA sequence were then simultaneously amplified using a polymerase chain reaction. The amounts of the amplified products were measured, and the amount of target viral RNA present before amplification was determined from the amounts of the amplified products.

2. The conception and reduction to practice occurred in the United States of America.

3. The date of conception prior to 21 August 1989 was determined by a draft patent application which describes the invention based on experiments conducted by us. Exhibit 1 is a

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The declarants further state that the above statements were made with the knowledge that willful false statements and the like are punishable by fine and/or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that any such willful false statement may jeopardize the validity of this application or any patent resulting therefrom.

Dated: _____ _____
George J. MURAKAWA

Dated: _____ _____
R. Bruce WALLACE

Dated: 12-02-05 _____
John A. ZAIA

Dated: _____ _____
John J. ROSSI

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George J. MURAKAWA

Dated: Dec. 1, 2005

R. Bruce Wallace

R. Bruce WALLACE

Dated: _____

John A. ZAIA

Dated: _____

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R. Bruce WALLACE

Dated: _____ _____
John A. ZAIA

Dated: _____ _____
John J. ROSSI

murappln

Exh. b+ 1

METHOD FOR AMPLIFICATION AND DETECTION OF RNA SEQUENCES

This application is a continuation-in-part of each of application Serial No. 355,296 filed May 22, 1989, (which is a continuation-in-part of application Serial No. 941,379, filed December 15, 1986), application Serial No. 143,045 filed January 12, 1988 (which is a continuation-in-part of application Serial No. 941,379), and application Serial No. 148,959 filed January 27, 1988. Application Serial Nos. 355,296, 143,045 and 148,959 are incorporated in this application by reference.

BACKGROUND

It is known to utilize the polymerase chain reaction (PCR) to amplify RNA and DNA sequences present in small samples. The amplification procedure can be simultaneously performed on more than one sequence in the same sample. The presence or absence of a specific sequence in the amplification products may be determined by oligonucleotide hybridization assays. See generally Mullis, U.S. patent 4,683,195.

Virus etiology generally and retrovirus etiology in particular is complex. See Varmus, Retroviruses, Science 240:1427-1435 (1988). Known PCR techniques, as applied rapidly diagnose or confirm potential retroviral positive patients, are of limited sensitivity, lack positive controls and may otherwise be unreliable. For example, persons who were seropositive but both virus culture-negative and PCR-negative are reported by Ou et al. Science 239:295-297 (1988). As a first explanation for this observation, Ou suggests that these persons may have contained an insufficient number of provirus copies to be directly detected by the PCR technique utilized.

SUMMARY OF THE INVENTION

This invention provides a PCR technique of improved sensitivity and which may include positive controls for determination of the presence or absence of a target sequence in viral RNA sample.

Increased sensitivity is provided by utilizing viral RNA as the original PCR template. The viral RNA is converted to complementary DNA which is then amplified.

The invention is useful to detect unique sequences in samples containing as few as 100 molecules of RNA. It can be used to detect retroviral RNA in samples from as little as 10 nanograms (ng) of total cellular RNA.

The practical application of the invention to clinical diagnosis of virus infected patients is apparent. Patients who harbor a viral genome but are not yet producing anti-viral antibodies may be diagnosed as uninfected by known screening methods. The invention enables detection of viral transcripts, such as those of the AIDS virus which may accumulate in the absence of viral protein translation during the early stages of infection. Clinical applications of the invention include the identification and quantification of viral RNA present in peripheral blood samples and ~~H-9 cells.~~ laboratory cell lines

DETAILED DESCRIPTION OF THE INVENTION

In general the method of the invention entails utilizing a sample RNA which has or may have a target viral sequence as a template for amplification by PCR. A first oligonucleotide primer for the target viral sequence is annealed to the template for extension through the target sequence to produce a first extension product having an RNA template strand and a DNA primer extension strand. The first extension product is denatured and the separated RNA template and DNA primer extension strands are

annealed, respectively to the first primer and to a second primer complementary to the DNA primer extension strand. The first and second primers are positioned for extension through the target sequence on the template and its complement on the primer extension strand. The first and second primers are extended to produce a second primer extension product which is denatured, the first and second primers are again annealed to the separated template and primer extension strands, and again extended and the resulting extension products denatured. The process is repeated for the number of cycles deemed appropriate to achieve the desired degree of amplification.

After the final round of amplification and denaturation, the product is analyzed, for example, by oligonucleotide hybridization assay to determine the presence or absence of a sequence indicative of the presence of the target sequence in the sample.

In the early cycles, e.g., the first five cycles after the production and denaturing of the first extension product, the amplification steps are conducted in the presence of both reverse transcriptase and the large fragment of DNA polymerase I (Klenow) or similarly functioning enzyme. Subsequent cycles may appropriately be conducted in the absence of reverse transcriptase. Ribonuclease A is preferably added after about 5 to about 7 cycles of DNA amplification to destroy residual RNA and reduces sequence complexity of the mixtures. In the preferred practice of the invention, both the first and second primer are present throughout the amplification procedure. Alternatively the second primer can be added at any stage of the process prior to the amplification of the denatured first extension product.

The process of the invention is useful to amplify and detect viral RNA from any source. It has particular application to the

detection and quantification of AIDS (HIV-1) virus and cytomegalovirus (HCMV).

For AIDS virus amplification and detection preferred first and second primers are synthetic oligonucleotides having the following sequences:

HIVA: 5'ATGCCGATTGTGCTTGGCTA 3'
HIVB: 5'TGAATTAGCCCTTCCAGTCC 3'

A preferred HIV-1 hybridization probe includes the sequence:

HIVC (PROBE): 5'AAGTGGCTAAGATCTACAGCTGCCT 3'

As applied to human cytomegalovirus (HCMV), a target for amplification is a region of the HCMV major IE gene (IE1) region between nucleotides 1154 and 1331. Oligodeoxyribonucleotides complementary to sequences in this region are used with RNA from HCMV infected cells, or from patient peripheral blood samples. Suitable oligonucleotide primers and probes have the following sequences:

HCMV	1154 5'	CGAGACACCCGTGACCAAGG 3'	1173
HCMVB	1311 3'	CTCTTTCTACAGGACCGTCT 5'	1330
HCMV (Probe I)	1182 3'	AAGGACGTCTGATACAACTCCTT 5'	1204

An additional amplification system is needed for detection of RNA from the transcriptase of late HCMV genes, which are important markers for active infection. For this purpose, sequences 866-1025 from the coding sequence of p64[1/] may be amplified. Suitable oligonucleotide primers and probes have the following sequences:

HCMVD	866 5'	AAAGAGCCCGACGTCTACTACACGT 3'	890
HCMVE	1001 3'	CTGGTCATGCAGTTCCACATGGACC 5'	1025
HCMV Probe II	941 3'	CGCGTGCTCGACCAAACGAGGTACCTCTTG 5'	970

EXAMPLE I

This example illustrates the amplification of *in vitro* synthesized RNA by the use of the plasmid pSP64-BH10-R3 (Biotech Research Laboratories, Inc.), containing the entire HTLV-III

1/ Ruger, B., et al. J. Virol. 61:446 (1987).

(HIV-1) virus excluding the LTRs, as the starting material for the following subclone vectors. A 1.1 kb BamHI restriction fragment including HIV-1 sequences 8052 to 9149 was subcloned in both orientations into the BamHI site of the transcription vector pGEM2 (Promega Biotec). The resulting plasmids, pGM92 (+ strand) and pGM93 (- strand), were digested with EcoR1 and transcribed with T7 RNA polymerase using a T7 transcription kit (BioRad Laboratories, Inc.).

10^{-1} pmol of RNA from pGM92 was subjected to 4, 5, 8 and 10 cycles of amplification. Amplification was performed using 1X and amplification buffer (10 mM tris-HCl, pH 7.5; 10 mM $MgCl_2$; 66 mM NaCl; 1 mM dithiothreitol), 1.5 mM of each dNTP, and 1.0 uM of each oligodeoxyribonucleotide (HIVA and HIVB, supra) in a final reaction volume of 100 ul. Samples were denatured by heating to 95°C for 2 minutes, spun in a microfuge for 5 seconds, cooled to 37°C for 2 minutes, at which time 1.0 ul of reverse transcriptase (2.0 units, BioRad), diluted in amplification buffer, was added for 2 minutes. Cycles 2-5 were performed as described above, except both reverse transcriptase and Klenow (0.5 units, Boehringer Mannheim) were added. In cycle 6, RNase A was added (0.45 ug) and only DNA pol I was used. All subsequent cycles of amplification were performed with only the presence of DNA pol I. After completion of the last cycle of amplification, samples were placed on ice and a 10.0 ul portion was electrophoresed in a 1.8% agarose gel. The DNA was transferred to Zeta probe (BioRad) using an alkaline blotting procedure[11] and prehybridized and hybridized as follows: The prehybridization reaction was performed at 65°C for 1 to 3 hours in 20 ml of 6X SSPE (1.0 M NaCl, 0.06 M $NaPO_4$, 0.006 M EDTA); 1.0% SDS; 0.5% rehydrated,

11/ Southern, E.M., J. Mol. Biol. 98, 503-517 (1975).

Reed + Mann - new DNA Prep

powder skim milk (Aldrich); and 10 ug per ml of sonicated, denatured salmon sperm DNA. The hybridization reaction was in 20 ml of the same buffer, except the salmon sperm DNA was omitted and replaced with 20 pmol of 5'-^{32}P-labelled oligodeoxyribonucleotide probe HIVC (ca. 3 x 10^8 cpm). Hybridization was for 1 hour to overnight at 65°C. The hybridized filter was washed with three 250 ml volumes of 6X SSC (0.95 M NaCl, 0.095 M Na Citrate), 0.1% SDS at 65°C for 5 minutes each, and autoradiographed at -70°C for 1 hour on Kodak XAR-5 film with an intensifying screen.

Densitometric scanning and integration of the peak areas were performed. This revealed a 3.81 fold level of amplification. Thus, if 21 cycles were performed with this template, and since only one strand is synthesized during the first cycle, we calculate the theoretical amplification would be over 400,000 fold.

EXAMPLE II

To test the sensitivity of amplification, samples in which 10^{-9}, 10^{-7}, and 10^{-5}, pmol of pGM92 RNA were used in repetitions of Example I. After 21 cycles of replication, bands from each of the samples can be detected after Southern blot hybridization. Since only one tenth of the reaction was used in the detection of the sample from only 10^{-9} pmol, this result shows that as few as 100 molecules of RNA are sufficient for detection after amplification.

EXAMPLE III

This Example demonstrates amplification of an RNA template in the presence of non-specific RNA. 5.5 ug of bovine rRNA was added to a reaction mixture as described in Example I containing 10^{-3} pmol of GM92 RNA. Specific amplification was seen at high efficiency.

EXAMPLE IV

This Example demonstrates that RNA isolated from HIV infected cells can be efficiently utilized for amplification and detection pursuant to this invention. Polymerase chain reaction using only 10 ng of total RNA from HIV infected H9 cells was performed as described in Example I. A specific hybridizing band, about two orders of magnitude lower than the 1.0 ug sample, was observed. To test if the amplification of the in vivo sample was from RNA or residual DNA contamination, a control sample in which RNase A was added prior to amplification was examined. In this experiment, no hybridization band was detected after prolonged autoradiographic exposure.

For identification and quantification purposes it is preferred to amplify the viral RNA sample simultaneously with at least one other RNA sequence to provide a positive control. In this way the risk of false negative data indicating the absence of viral RNA in, for example, peripheral blood and H-9 cell samples may be reduced.

To provide such positive controls at least one synthetic RNA sequence is amplified simultaneously with the RNA sample. In clinical practice the sample usually is from virus infected T-4 lymphocytes of ~~H-9 cells~~ present in a peripheral blood sample. Such embodiments entail use of a plurality of first and second primer pairs. One pair is provided for each RNA sequence to be amplified. The amplification procedure is otherwise accomplished as previously described.

The cell population primarily affected by a virus, e.g., the AIDS virus, is the T-4 lymphocyte population which, like other T-cells express the T-cell receptor.

Accordingly, a suitable second primer pair includes oligonucleotides to amplify a sequence which is unique to the T-cell receptor. Although other such unique sequences may be selected, preferably such a primer is effective to amplify the constant region of the beta chain of the relevant virus T-cell receptor. For HIV-1, T-cell receptor the following primers A and B are preferred:

T-Cell Receptor A: 5'GTCCACTCGTCATTCTCCGA 3'
T-Cell Receptor B: 5'TCAAGACTCCAGATACTGCCT 3'

A preferred T-cell receptor hybridization probe includes the following sequence:

T-Cell Receptor C (PROBE): 5'CAGAAGGTGGCCGAGACCCTCAGGC 3'

Another primer pair is effective to amplify an RNA sequence present, preferably ubiquitously, in all of the cells of a patent sample such as a peripheral blood sample or ~~of an H-9 cell sample~~, even when the T-cell count is low. Preferably such primer pairs amplify beta actin sequence. Synthetic oligonucleotides comprising the following sequences are preferred:

Beta Actin A: 5'CTCATTGCCAATGGTGATGACCTG 3'
Beta Actin B: 5'GCTATCCCTGTACGCCTCTGGC 3'

A preferred beta actin hybridization probe includes the sequence:

Beta Actin C (PROBE): 5'CGGTGAGGATCTTCATGAGGTAGTC 3'

An additional aid to quantitation of virus levels in patient samples is provided by a reference RNA which can be amplified and detected by the same oligonucleotides used for authentic virus RNA samples.

Such a reference RNA may be a "minigene" or a "maxigene" formed by a multi-base pair insert into or deletion from a unique site. For example a preferred reference RNA includes a 22 base pair insert into the KpnI site of the HIV-1 3' ~~ORF~~ nef region of the pGEM92 clone described in Example I. An insert of sequence:

~~5'GACACAAGGCTACTTC**GGTAC**.**CATGG**TGTGTTCCGATGAAGC5'~~ is appropriate. The underlined sequences present in the AIDs virus bracket the 22 base pair insert.

The transcription product of this clone is 22 bases longer than the authentic HIV-sequence but still hybridizes with the 25-mer probe HIVC. It is therefore distinguishable by size from the authentic viral product.

Such "minigenes" and "maxigenes" provide an internal control and an additional aid to quantitation. Because the quantity of "maxigene" RNA originally included in the amplification reaction is known, the amount of signal obtained from this amplification product can be related to the signal obtained from the patient sample. Hence, the relative quantitation of the original amount of authentic HIV-1 in the patient sample is also provided by this construct.

Similar procedures can be used as a quantitative assay of HCMV sequences. A segment of the cDNA derived from the major IE gene IE1 is subcloned into the transcription vector pTZ18U (BioRad), and includes nucleotides 1185-1331. A small insertion accomplished either by cloning or by site directed mutagenesis is made in this segment which permits distinction between the PCR-amplified viral RNA and cellular amplified transcripts. By including a fixed amount of this plasmid HCMV RNA or DNA in every sample to be amplified, it is possible to measure the amount of viral DNA or RNA using the *in vitro* sample as an internal standard.

To provide appropriate signals either the primers or the probes are labelled, e.g., with an isotope such as P^{32} or a fluor~~escent.~~ Preferably, the probes are labelled.

Amplification using an oligonucleotide primer containing the T-7 RNA polymerase (BioRad Laboratories) increases the sensitivity of detection. The following HIV T7 sequence is illustrative:

HIV T7 - 5'TTAATACGACTCACTATAGGCATGCTGATTGTGCCTGGCTA 3'.

For purposes of identification and quantification, the amplification products may be electrophoresed in a gel, e.g., agarose or 6% polyacrylamide, 7 M. urea gel. Labelled probes complementary to each of the amplified sequences are used sequentially. Hybridization of the probes with amplification products other than of authentic viral sequence, e.g., HIV or HCMV provides positive controls thus minimizing the possibility of false negative data regarding the authenticity of the original sample. More particularly, if the authentic, e.g., HIV probe yields negative data, but one or both the T-cell receptor and beta actin probes yield positive data, the conclusion may be feasibly drawn that the original sample was viable notwithstanding the negative HIV probe result.

EXAMPLE IV

Amplification is performed using 1X amplification buffer (10 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 66 mM NaCl; 1 mM dithiothreitol), 1.5 mM of each dNIP). To this buffer, about 1 mM total peripheral blood lymphocyte RNA from an AIDS infected patient in about 1.0 mM of each of the priming nucleotides HIVA, HIVB T-cell receptor A and T-cell receptor B are added providing a final reaction volume of approximately 100 μl. The sample is heated at 95°C for 2 minutes, centrifuged for 5 seconds, cooled to 37°C for about 2 minutes at which time 1.0 μl of AMV reverse transcriptase (Life Sciences or BioRad Laboratories) diluted in the amplification buffer were added and incubation was continued for 2 minutes at 37°C. A second amplification cycle was

performed in like manner. Thereafter the final 28 rounds of amplification were accomplished using a buffer consisting of 2.5 units of Thermus aquatus DNA polymerase (Perkin-Elmer Cetus or New England Biolabs): 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each dNTP, and 50 pmoles of each primer in a final volume of 50 microliters overlain with 10 microliters of paraffin oil. The polymerizations are carried out from 1 to 2 minutes at 65°C, with 1 minute of denaturation at 95°C, and 1 minute of annealing at 37°C.

After completion of the last cycle of amplification, the products are placed on ice and a 10 μl portion was electrophoresed in a 1.8% agarose gel. The DNA was transferred to Zeta probe (BioRad) using an alkaline blotting procedure and prehybridized and hybridized as follows: The prehybridization reaction was performed at 65°C for 1 to 3 hours in 20 ml of 6X SSPE (1.0 M NaCl, 0.06 $NaPO_4$, 0.006 M EDTA); 1.0% SDS; 0.5% rehydrated, powder skim milk (Alba); and 10 μg per ml of sonicated, denatured salmon sperm DNA. The hybridization reaction was in 20 ml of the same buffer, except the salmon sperm DNA was omitted and replaced with 20 pmol of 5'-^{32}P-labelled oligodeoxyribonucleotide HIVC (ca. 3 x 10^8 cpm). Hybridization was for 1 hour to overnight at 65°C. The hybridized filter was washed with three 250 ml volumes of 6X SSC (0.95 M NaCl, 0.095 M Na Citrate), 0.1% SDS at 65°C for 5 minutes each, and autoradiographed at -70°C for 1 hour on Kodak XAR-5 film with an intensifying screen.

Each of the HIVC and T-cell receptor C probes is used separately and sequentially. After the results with the HIVC probe are obtained, that probe is stripped from the filter by treatment with 100C 0.1 X SSC, 0.1% SDS, two times for 15 minutes each. The filter is then rehybridized to the T-cell receptor C probe.

Bands from each of the authentic HIV and T-cell receptor samples are detected after Southern Blot hybridization.

Figure 1 exemplifies simultaneous priming with HIVA and B and T-cell receptor A and B synthetic oligonucleotides. Figure 1A shows the results with HIVC probing. Figure 1B shows the results with T-cell receptor probing.

Figure 2 shows that HIV oligonucleotide primers and probes have homology with the human cytomegalovirus (HCMV) suggesting that HIV-1 has homology with an actively transcribed region of HCMV.

EXAMPLE V

Example I is repeated with the exception that the primer pair beta actin A and beta actin B is included in the amplification reaction mixture.

The amplification products are analyzed separately and sequentially by probes which hybridize with authentic viral RNA, the amplified T-cell receptor RNA sequence and the amplified beta actin A sequence. Bands from each such sequence are detected after Southern blot hybridization.

EXAMPLE VI

Example I is repeated with the exception that the maxigene primer is included in the reaction mixture.

Kits contemplated by the invention include self-contained appropriate quantities of primers and probes for use in the practice of the invention.

A typical kit for the detection and quantification of HIV-1 virus in a patient peripheral blood sample includes vials or similar separate containers filled with, for example, __ ml of each of HIV-1 A and HIV-1 B in __% solution in _____ and __ ml of HIV-C in __%. Reference RNA in ___% solution in _____ is included in an additional container. Such kits

20 pmoles/microliter (in sterile H_2O) each of HIVA, HIVB or HIVC. A reference RNA (~10,000 copies/microliter) in prepared in sterile DEPC treated water

include reagents and instructions necessary to conduct the appropriate amplification and hybridization procedures.

WE CLAIM:

1. The process for detecting the presence or absence of a viral RNA in a sample which comprises

(i) utilizing the sample as a template for hybridization with a first primer for a target viral RNA sequence present in substantially all transcripts of a virus, said primer being present in excess and positioned for extension through said target sequence;

(ii) extending said first primer to provide a double stranded first extension product having an RNA template strand including said target sequence and a DNA primer extension strand complementary to said target sequence;

(iii) denaturing said first extension product to provide a mixture containing said first primer and said separate RNA template and DNA primer extension strands;

(iv) providing, in the step (iii) mixture a second primer for extension through said sequence in said DNA primer extension strand which is complementary to said target sequence;

(v) annealing said first and second primers respectively to said separated RNA template strands and DNA primer extension strands;

(vi) extending said annealed primers;

(vii) denaturing the extension products produced in step (vi);

(viii) subjecting the product of step (vii) to hybridization with a labelled oligonucleotide probe for said target viral sequence or for a sequence complementary to said target sequence.

2. A process as defined by claim 1 in which steps (v), (vi), and (vii) are repeated prior to step (viii).

3. A process as defined by claim 1 or claim 2 in which said second primer is present during steps (i), (ii), and (iii).

4. A process as defined by claims 1, 2, or 3 in which said viral RNA is human immunodeficiency virus (HIV) RNA or human cytomegalovirus (HCMV).

5. A process as defined by claims 1, 2 or 3 for detecting the presence or absence of viral RNA in a human H-9 cell or peripheral blood sample.

6. A process as defined by claims 1, 2, or 3 for detecting the presence or of HIV-1 or HCMV in a human H-9 cell or peripheral blood sample.

7. A process as defined by claims 1, 2, or 3 in which said viral RNA is HIV-1 and in which

(i) said target viral sequence is the BamHI-Sst-I fragment within the 3' ORF region of the HIV-1;

(ii) said first primer includes the sequence 5'TGAATTAGCCCTTCCAGTCC encoding nucleotides 8677 to 8658.

(iii) said second primer includes the sequence 5'ATGCTGATTGTGCCTGGCTA, encoding nucleotides 8538 and 8547, and

(iv) said probe includes the sequence 5'AAGTGGCTAAGATCTACAGCTGCCT, encoding nucleotides 8642 to 8618.

8. The process for detecting the presence or absence of a human cytomegalovirus (HCMV) in a sample which comprises:

(i) utilizing the sample as a template for hybridization with a first primer for a target viral RNA sequence present in all transcripts of said HCMV, said primer being present in excess and being positioned for extension through said target sequence;

(ii) extending said first primer to provide a double stranded first extension product having an RNA template strand including said target sequence and a DNA primer extension strand including a sequence complementary to said target sequence;

(iii) denaturing said first extension product to provide a mixture containing said first primer and separate RNA template and DNA primer extension strands;

(iv) providing in the step (iii) mixture a second primer for extension through said sequence in said DNA primer extension strand which is complementary to said target sequence;

(v) annealing said first and second primers respectively to said separated RNA template strands and DNA primer extension strands;

(vi) extending said annealed primers;

(vii) denaturing the extension products produced in step (vi);

(viii) adding a labelled oligonucleotide probe for said target viral sequence to the product of step (vii).

9. A process as defined by claim 8 in which steps (v), (vi) and (vii) are repeated prior to step (viii).

10. A process as defined by claim 8 in which said second primer is present during steps (i), (ii) and (iii).--

11. A process as defined by claim 8 in which said first primer includes the sequence 5' CGAGACACCCGTGACCAAGG 3'encoding nucleotides 1154 to 1173;

said second primer includes the sequence
3' CTCTTTCTACAGGACCGTCT 5' encoding nucleotides 1311 to 1330, and

said probe includes the sequence
3' AAGGACGTCTGATACAACTCCTT 5'.

12. A process as defined by claim 8 for detecting the presence or absence of RNA from a human cytomegalovirus late gene transcript in a sample in which said target viral sequence includes nucleotides from the coding sequence of p64, said first primer includes the sequence 5' AAAGAGCCCGACGTCTACTACACGT 3', said second primer includes the sequence 3'

CGCGTGCTCGACCAAACGAGGTACCTCTTG 5′, and said probe includes the sequence 3′ CGCGTGCTCGACCAAACGAGGTACCTCTTG 5′.

13. A process which comprises:

(i) providing an amplification reaction mixture of a viral RNA, including a target sequence, a primer for annealing to said target sequence, reverse transcriptase, and a second primer for the annealing to and for extending across the DNA complement of said RNA target sequence;

(ii) annealing said first primer to said target sequence;

(iii) extending said first primer across said target sequence to provide a first extension product having an RNA target sequence strand and a DNA primer extension strand;

(iv) denaturing said first extension product to provide separate RNA target sequence and DNA primer extension strands;

(v) annealing said first and second primers to said separate RNA target sequence strands and DNA primer extension strands;

(vi) extending said annealed primers to produce a second extension product;

(vii) denaturing said second extension product;

(viii) repeating steps (ii) through (vii);

(ix) subjecting the amplification product after step (viii)to hybridization with a labelled oligonucleotide probe for said target sequence or for a sequence complementary to said target sequence.

14. A process as defined by claim 1 or 2 in which

(a) at least one synthetic RNA sequence which does not include said target sequence of which includes substantially more or less nucleotides than said target sequence is subjected, with said sample to polymerase chain reaction

amplification under conditions appropriate to simultaneously amplify said target sequence and said reference sequence;

(b) the amplification products of step (a) are denatured and thereafter separately and sequentially subjected to hybridization conditions with oligonucleotide probes for said target sequence and said reference sequence.

15. A process for minimizing false negative data in the identification of a target viral RNA sequence in a peripheral blood or H-9 cell sample which comprises:

(i) selecting said target viral RNA sequence;

(ii) simultaneously subjecting

(a) said sample and

(b) at least one synthetic RNA sequence which does not include said target sequence or which includes substantially more nucleotides than said target sequence

to polymerase chain reaction amplification under conditions appropriate to simultaneously amplify said target sequence if present in said sample and said reference sequence;

(iii) denaturing the amplification products produced by step (ii);

(iv) subjecting said denatured amplification products of step (iii) to hybridization conditions separately and sequentially with probes homologous to said target sequence and to said reference sequence,

each of said probes being removed from a sequence with which it is hybridized prior to the separate and sequential subjection of said amplification products to hybridization with another of said probes;

(v) determining whether said amplified target and reference sequences hybridized with said probes homologous therewith.

16. A process as defined by claim 15 in which the reference nucleotide sequence utilized in step (ii) is

(i) a sequence present in the T-cell receptor expressed by cells affected by the virus containing said viral RNA;

(ii) a preselected RNA sequence present in substantially all of the cells of said sample,

(iii) a sequence including said target and constructed by a multi-base insertion into a site in said viral RNA preselected with respect to said target sequence;

(iv) a beta actin sequence.

17. A process as defined by claim 15 in which said target viral sequence is located within the 3′ ORF region of HIV-1 and in which the reference sequence utilized in step (ii) is located in the constant region of the beta chain of the T-cell receptor expressed T-cells affected by HIV-1.

18. A process as defined by claim 17 in which the reference sequence utilized in step (ii) is a beta actin sequence.

19. A process as defined by claim 17 in which the reference sequence utilized in step (ii) is a sequence formed by inserting a multi-base pair sequence into the 3′ ORF region of HIV-1.

20. A process as defined by claim 15 or 17 in which at least one of the primers utilized in conducting the polymerase chain reaction in step (ii) includes the T-7 RNA polymerase sequence.

21. A process as defined by claim 15 in which said target viral sequence is in the HCMV major immediate early (IE) gene.

22. A process as defined by claim 15 in which said target viral sequence comprises RNA from the transcription of late HCMV genes.